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HPTLC fingerprinting of *Azadirachta indica* leaf and bark extracts and their antimicrobial activity against *Escherichia coli*.

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ABSTRACT

The *Meliacea* family includes *Azadirachta indica* L., popularly referred to as "neem," which is extensively accessible throughout the Indian subcontinent. The neem tree is well-known for its many medicinal uses, including as an insecticide, pesticide, antidiabetic, antibacterial, and antiparasitic. The plant's leaves are frequently employed in "kwath" and "churna," two Ayurvedic preparations. It is crucial to standardise and record the quality criteria of the plant leaves because of their great medicinal potential and to evaluate the scientific research on *Azadirachta indica's* utilisation. Aqueous extracts of the plant's dried leaves and bark were tested for antimicrobial activity against gram-negative *Escherichia coli* bacteria. Using a solvent system of toluene: ethyl acetate: formic acid 5:4:0.2, the ethanolic extract of dried powdered leaves and bark was assessed by HPTLC fingerprinting, which shows the identities of the chemicals included in the extracts. By measuring the width of the zone of inhibition against the bacteria using the agar well diffusion technique, the antimicrobial activity of the concentrated extracts was assessed. In both aqueous extracts, the bark and leaves of *A. indica* showed antibacterial action against *E. coli.* The different zones of inhibition with concentration changes demonstrated that the antibacterial activity of *A. indica* extracts was concentration-dependent. According to the study's findings, *Azadirachta indica* leaves can be used as an alternative medical system to treat a variety of microbial illnesses.

Keywords: HPTLC , Antimicrobial, *Azadirachta indica*, *Escherichia coli* , Aqueous extract.

INTRODUCTION

Human civilisation has relied on nature to provide its requirements from the beginning. Humans use natural items, particularly those produced from plants, as medicines to treat and cure various illnesses. The global popularity of Ayurveda is evidence of the significance of plants. Plants are becoming a new source of possible lead compounds in an era when synthetic medications are failing to treat diseases and rapidly increasing microorganisms' resistance to them. [1–3] The extensive collection of constitutively generated secondary metabolites in plants is well-known.

These comprise a range of phytochemicals generally classified as glycosides, steroids, terpenoids, phenols, alkaloids, and their derivatives. Since secondary metabolites are known to have medicinal and pharmacological properties, researchers are looking for strong chemical moieties. [4] The application of contemporary analytical methods for the separation, identification, and structural elucidation of the isolated phytochemical substances has significantly aided phytochemical research.

Due to its ease of cultivation in tropical and sub-tropical woods, *Azadirachta indica* L. (Fam. Meliaceae), generally known as "neem," is widely distributed over the Indian subcontinent and most African nations. The neem tree is well-known for its many medicinal uses and for treating various illnesses, including fever, stomach infections, and acne. According to Ayurveda, neem balances the human body's four doshas- pitta, vata and kapha. In addition to being used as a mosquito repellent, neem oil is believed to treat toothaches.

Veppampoo charu, a soup-like meal, is prepared in Tamil Nadu using the delicate shoots of the neem tree. "Begun bhaja," a well-known Bengali delicacy, is made using neem blossoms. In Ayurveda, different churna and kwath are made from powdered neem leaves. Recent studies have demonstrated the antifungal and antibacterial properties of A. indica leaves and its anti-inflammatory, antiarthritic, antipyretic, hypoglycemic, anti-gastric ulcer, and anticancer properties. [5–8].

Chemical components such as salanin, nimbin, and nimbidin are responsible for the high bioactivity of these neem products [9]. The leaves are effective against ophthalmia, biliousness, skin conditions, cough, asthma, piles, and

tumours. They are also alexiteric, insecticidal, and anthelmintic [10]. High-performance thin-layer chromatography (HPTLC) is crucial for evaluating the quality of herbal medications and formulations' quality. It makes it possible to analyse many substances effectively and economically. Aqueous extracts of *Azadirachta indica* leaves and bark are tested for HPTLC figure printing of chemical contents and for examining the antibacterial activity of the extracts on *Escherichia coli* due to their many therapeutic uses.

MATERIALS AND METHODS

Collection of Plant Materials

In January 2025, *A. indica* leaves and bark were gathered at the Ganga Devi Mahila Mahavidyalaya. The plant material was first soaked in regular water for two to five minutes to get rid of any dirt. It was then rinsed twice with deionised water. Following washing, the material was allowed to dry at room temperature in the dark while being closely monitored to avoid contamination. In the end, an electric grinder was used to grind it. After that, the powdered material was kept for future research in sealed vials.

Test organisms

The antimicrobial activity was assessed using two bacterial cultures: *E. coli* (MTCC40) from the Microbial Type Culture Collection and Gene Bank (MTCC), a national facility founded in 1986 and supported by the Government of India's Department of Biotechnology (DBT) and Council of Scientific and Industrial Research (CSIR).

Preparation of Bacterial Isolates

According to Beyene and Tsegaye (2011)[11], the bacteria were kept at 37˚C in a controlled acidity environment while being fed the right amount of food to encourage their growth.

Inoculum Preparation

Saline & 0.5 McFarland turbidity principles were utilised to compare turbidity because this study employed the direct state suspension method. Saline balanced the turbidity until it matched the 0.5 McFarland turbidity measurements. To complete this, the suspension and the 0.5 McFarland turbidity gauges were held in front of a light source against a white foundation with distinct dark lines [12].

Inoculation

For *E. coli,* Muller-Hinton agar plates were used. The agar surface was carefully checked for excessive wetness before inoculation, and the plates were checked for excessive dryness (a wrinkled surface indicates excessive dryness). The suspension of microscopic organisms was put onto a sterilised cotton swab. The abundant inoculum was released by inserting the swab into the suspension tube for the microscopic organisms. Before using the test plant, the media was inoculated by swabbing the agar surfaces in two directions at a 90-degree edge to each surface and the third line at a 45-degree edge. This was done for 20 minutes to promote assimilation of the abundant inoculum [13].

Disc Diffusion Method

The bactericidal qualities were tested using the disc diffusion method. The extract was applied to the disc, allowed to permeate the disc, and then set on the bacterial agar medium [14]. Dimethyl sulphoxide (DMSO) and sterile water were employed as negative controls to compare the results with those of experimental ethanol and water extracts, respectively. At the same time, amikacin 5 µg standard discs were used as the positive control against *E. coli.*

Antimicrobial Activity Determination

The aqueous extracts of *A. indica* were evaluated on *E. coli* culture strains on agar plates at various concentrations (10 mg/mL, 20 mg/mL, and 30 mg/mL). Zones of inhibition during incubation demonstrated the studied pathogenic organisms' susceptibility to aqueous and methanolic extracts. The zone scale was also used to measure the zone of inhibition's (ZOI) diameter in millimetres [15]. The zone of inhibition was measured three times for each extract concentration to reduce error, and the mean was noted. After that, the statistical analysis was carried out. The agar-disc diffusion method was used to calculate the crude extract's Minimum Inhibitory Concentration (MIC). According to Margaret, P., et al. (2020)[16], MIC is the lowest concentration at which a distinct zone of microbial growth inhibition will be visible.

High Performance Thin Layer Chromatography (HPTLC)

High-Performance Thin-Layer Chromatography (HPTLC) was conducted using pre-coated silica gel 60 F_{254} HPTLC plates (50 × 100 mm; Merck, Darmstadt, Germany) as the stationary phase. Samples of the test substance (labelled KA1) were prepared in ethanol and applied to the plate in varying volumes (5.0, 6.0, 8.0, and 9.0 µL) using a CAMAG Linomat 5 automatic applicator (Serial No. 100632, CAMAG, Muttenz, Switzerland). Sample bands were applied 8.0 mm from the lower edge of the plate, with a band length of 8.0 mm and an inter-track distance of 10.4 mm. The dosage speed was set at 100 nL/s, and a pre-dosage volume of 0.20 µL was used to ensure accurate sample delivery.

Chromatographic development was performed in a twin-trough glass chamber (20 × 10 cm) pre-saturated for 20 minutes with a mobile phase as mentioned in the report. Using appropriate filter paper liners to ensure consistent vapour phase conditions, saturation was carried out. Plates were developed up to a solvent front distance of 70 mm, followed by drying at ambient room temperature for 5 minutes.

Densitometric evaluation of the plates was conducted using a CAMAG TLC Scanner 3 (Serial No. 140507), operated under vision CATS software version 3.2.23095.1 (CAMAG, Switzerland). Scanning was performed in absorbance mode at multiple wavelengths—254 nm, 366 nm, 430 nm, and 480 nm—using deuterium and tungsten lamps. The scanner was configured with automatic detector mode, a scanning speed of 20 mm/s, a resolution of 100 µm/step, and a slit dimension of 5 × 0.45 mm (micro).

Data acquisition and processing included Savitzky–Golay smoothing (window size 7), baseline correction using the lowest slope method, and peak detection using a Gaussian legacy algorithm with sensitivity set to 0.1, peak separation of 1, and a threshold of 0.1. Integration was carried out over an RF range of 0.00 to 1.00. Each sample volume yielded consistent peak patterns with reproducible RF values, supporting the method's robustness. All operations were executed per CAMAG standard practices and analytical guidelines for HPTLC as described in the literature [17, 18, 19].

Toluene: ethyl acetate: formic acid 5:4:0.2 was employed as the final solvent system, as it gave the components a good resolution. The chromatographic plate was scanned at 254 nm to obtain a fingerprint of the extract. 100 mg dried extract of *Azadirachta indica* was accurately weighed and dissolved in 100 ml of ethanol to get a 1 mg/ml concentration.

RESULTS AND DISCUSSION

Antibacterial Activity of Aqueous Leaf and Bark Extracts of *A. indica* against *E. coli*

The aqueous bark extract at 30 mg/ml produced the highest zone of inhibition (7.88 ± 1.3 mm) compared to the leaf extract (7.23 ± 0.27). The mean zone of inhibition for amikacin (control) was 14.24 ± 0.32 mm. The aqueous bark extract's zone of inhibition against *E. coli* was 10 mg/ml(2.8 ± 2.14 mm), but the aqueous leaf extract had a zone of inhibition of 10 mg/ml (2.5 ± 2.02 mm).

Table 1. Mean zone of inhibition of aqueous and methanolic bark extracts against *E. coli.*

Concentration of extract	Aqueous Bark extract Mean zone of inhibition (mm)	Aqueous leaf extract Mean zone of inhibition (mm)	Amikacin (control) mean zone of inhibition (mm)
10 mg/mL **20 mg/mL** **30 mg/mL**	2.8 ± 2.14 5.2 ± 1.4 7.88 ± 1.3	2.5 ± 2.02 4.9 ± 0.18 7.23 ± 0.27	14.24 ± 0.32

*- MZI of aqueous bark and leaf extracts against *E. coli.*

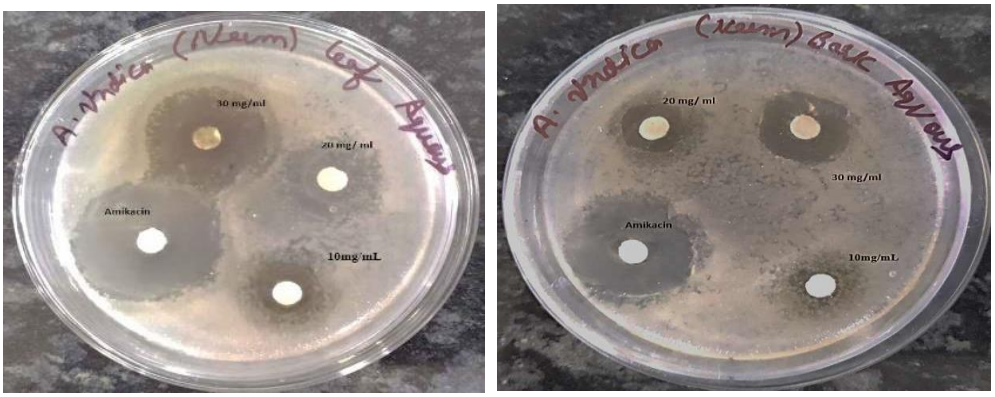

Figure 2: Zone of inhibition of aqueous leaf and bark extracts against *E. coli*.

High Performance Thin Layer Chromatography (HPTLC) of Aqueous leaf and bark extracts of *A. indica*

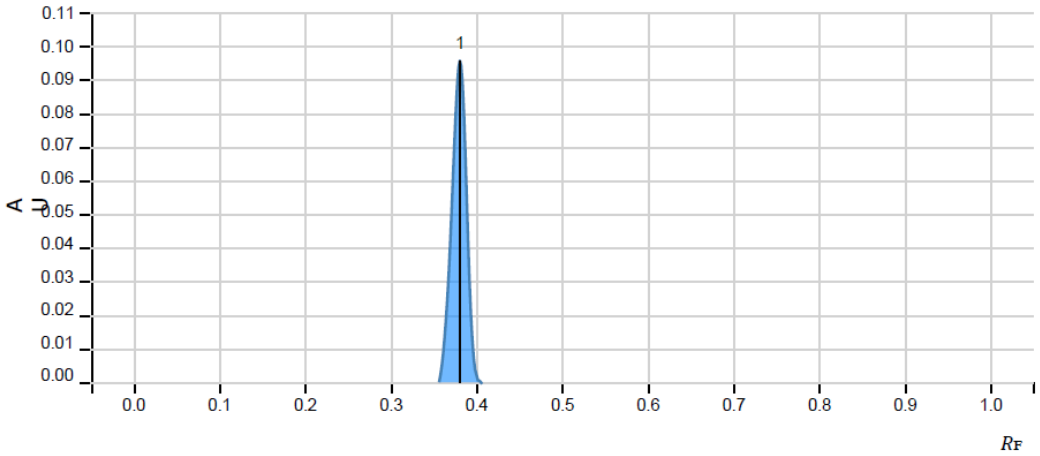

Figure 3: HPLC Chromatograms of Azadirachtin(standard) at Volume 4.0 µL

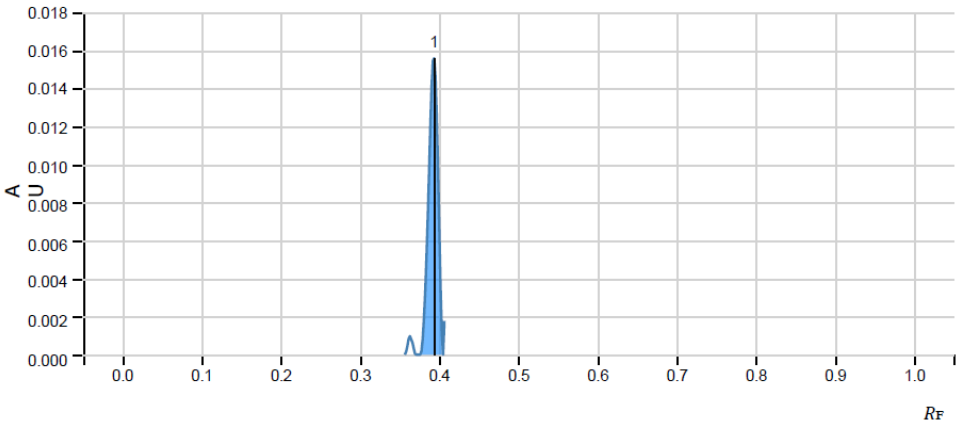

Figure 4: HPLC Chromatograms of at Volume 4.0 µL of aqueous leaf extract of *Azadirachta indica (AIL)*

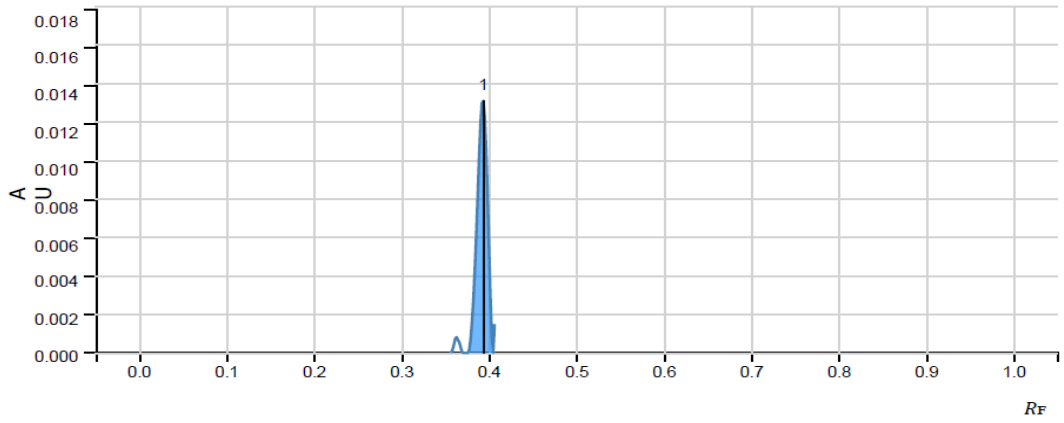

Figure 5: HPLC Chromatograms of at Volume 4.0 µL of aqueous Bark extract of *Azadirachta indica (AIB)*

Peak For	Start		Max			End		Area	
	RF	H	*RF*	H	%	*RF*	H	A	%
A	0.356	0.0000	0.381	0.0956	100.00	0.406	0.0000	0.00192	100.00
AIL	0.376	0.0000	0.394	0.017	100.00	0.405	0.0000	0.00045	100.00
AIB	0.376	0.0000	0.394	0.0156	100.00	0.405	0.0000	0.00022	100.00

Table 2: *Azadirachta indica* extract at 254 nm. Azadirachtin(standard), AIL, AIB Plate.

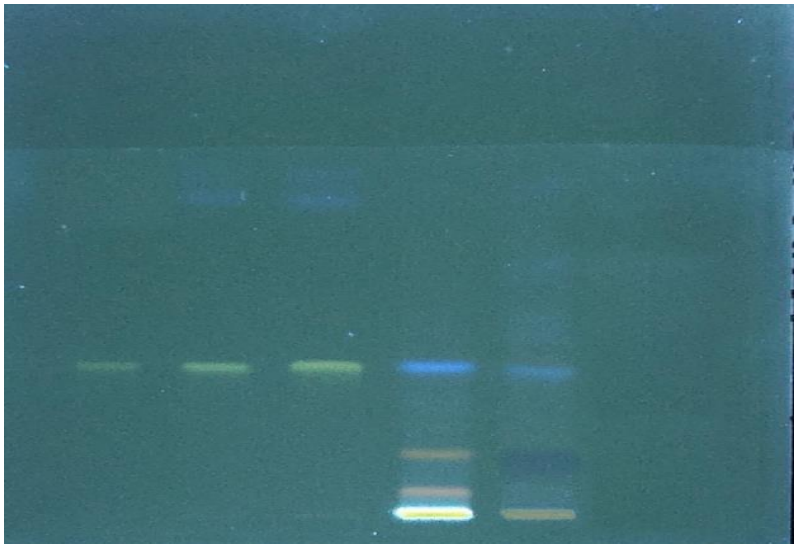

Figure 5: Chromatography of *Azadirachta indica* extract at 254 nm. Azadirachtin(standard), AIL, AIB Plate, Tracks from left to right, Track, Azadirachtin (Standard), Track 2 Azadirachtin (Standard), Track 3 Azadirachtin (Standard), Track 4 AIL, Track 5 AIB

The overlay spectra of HPTLC chromatographs of *Azadirachta indica* extract, as shown in Figures 3 to 5, exhibit Rf values of 0.356, 0.376 and 0.376 for Azadirachtin(standard), AIL, and AIB for which confirms the presence of azadirachtin and other phyto-constituents in *A indica* plant aqueous extract. As the extract was antioxidant antioxidant-rich, so was tested with known antioxidant compounds by TLC analysis. Finally, through HPTLC analysis, the presence of Azadirachtin was detected in the extract.

Chemical compounds that have a specific physiological effect on the human body are responsible for the secondary metabolites' therapeutic qualities. Some of these chemicals include alkaloids, glycosides, steroids,

flavonoids, fatty oils, resins, mucilage, tannins, gums, and calcium and phosphorus for bodybuilding, cell growth, and replacement [20].

Amikacin 5 µg, the positive control, provided an average zone of inhibition of 14 mm with p-value = 0.025. In contrast, the highest concentration of 30 mg/ml of aqueous leaf and bark extracts demonstrated an average zone of inhibition of 7.23 ± 0.27 mm and 7.88 ± 1.3 mm, respectively. Since Amikacin had 100% of its active component and was in its purest antibacterial form, there was a statistically significant difference between the three outcomes. The aqueous extracts did not differ statistically at 30 mg/ml. The aqueous bark extract performed statistically considerably better than the aqueous leaf extract at different doses.

The methanolic extract's activity against *E. coli* was 8 mm, whereas the aqueous extract exhibited no activity, according to Susmitha et al. (Susmitha, S., et al, 2013). However, as solvents with varying polarity were utilised, the observed variation in inhibitory zones may result from variations in the extracts' phytochemical content. As this study showed, different polarities had an impact on the kinds of phytochemicals that were extracted and, consequently, the plant extract's medicinal properties. Furthermore, variations in harvesting seasons, rainfall patterns, and geographic locations may be the cause of observed discrepancies among the published research. The methanolic extract of *A. indica* had a minimum inhibitory concentration (MIC) of 20 mg/mL, whereas the aqueous extract had a MIC of 10 mg/mL. The MIC for *A. indica* methanolic extract against *E. coli* was found to be 83.3 ± 29.0 mg/ml in a study by Nigussie et al. [21]. Shu'aibu et al.'s investigation, however, found that the petroleum ether extract of *A. indica* exhibited a minimum inhibitory concentration (MIC) of 100 µg/mL against *E. coli,* although the methanolic extracts showed no action at that concentration [22]. This indicates that the polarity of the extraction solvent has a major impact on *A. indica* activity.

Conclusion

The methanolic and aqueous extracts of the leaf and bark of *A. indica* showed antibacterial activity against *E. coli*. Compared to the methanolic extract, the aqueous extract exhibited superior antibacterial activity. As seen by the different zones of inhibition with concentration changes, the antibacterial activity of *A. indica* extracts was concentration-dependent. HPTLC chromatographic analysis will also add value to the research community. Through high-throughput screening, it can be concluded that *Azadirachta indica* leaves and bark are rich in antioxidants and have potent antimicrobial activities. This study paves the way for the advanced monograph development of herbal products and formulations.

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Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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